

Gonococcal serology

A comparison of three different tests

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SUMMARY Three serological tests for the detection of gonococcal antibodies were compared: an enzyme-linked immunosorbent assay (ELISA), an indirect haemagglutination reaction (IHA), and a gonococcal complement-fixation test (GCFT). The ELISA was performed with gonococcal pili of a Rotterdam strain (1443) as antigen, the IHA with pilus antigen of an American strain (2686, Buchanan), and the GCFT with whole gonococci of a single strain (46695, Oliver) as antigen. The tests were performed on sera from the same groups of Dutch patients; samples of sera were taken at the first examination and generally 11-22 days later.

The ELISA and the IHA were more sensitive than the GCFT. The specificity of the tests was equal in low-risk groups, but the GCFT was slightly more specific in high-risk groups. The ELISA and the IHA did not differ in sensitivity and specificity. The agreement between the ELISA and IHA for patients with uncomplicated gonorrhoea was low ($\kappa = 0.44$), but the agreement between the GCFT and the two pilus assays was less ($\kappa = 0.26$ and 0.20). The sensitivities were highest for sera from patients with oropharyngeal gonorrhoea or with gonococcal complications; again the ELISA and the IHA were more sensitive than the GCFT.

Introduction

At present, the most interesting gonococcal antigens for use in serological tests and as vaccine are pili and outer-membrane protein complexes.¹⁻⁵ Buchanan and colleagues² were the first to report promising results obtained with a radioimmunoassay (RIA) using gonococcal pili of strain 2686 (USA) as antigen. The sensitivity was 44% and 86% in sera from men and women with uncomplicated gonorrhoea respectively, Reimann and colleagues⁶ obtained almost equally good results with an indirect haemagglutination reaction (IHA) using a mixture of gonococcal pili of two strains.

In this study we compared an enzyme-linked immunosorbent assay (ELISA) using gonococcal pili of a Rotterdam strain (1443) with the IHA using pili of Buchanan's strain 2686 and the gonococcal

complement fixation test (GCFT) routinely used in the Netherlands, in which whole gonococci of a single strain (Oliver) were used as antigen.

Patients and methods

DIAGNOSIS

Between September 1977 and July 1978 sera were obtained from patients with and without gonorrhoea attending an STD clinic at the University Hospital in Rotterdam. Samples were taken from the cervix, urethra, rectum, and oropharynx of women and from the urethra, oropharynx, and rectum (from the latter site only if indicated) of men. Each specimen was cultured on selective Thayer-Martin medium⁷ and on chocolate agar medium without antibiotics. Oxidase-positive colonies were identified as gonococci by means of Gram-stain microscopy and sugar fermentation reactions. If cultures of specimens collected at the first and a second attendance (after 1-2 weeks) were negative, the patient was considered not to have gonorrhoea.

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PATIENT GROUPS

Generally patients were included in the study only if two consecutive serum samples could be obtained at an interval of 11-22 days. They were then assigned to one of two groups.

Patients with gonorrhoea

This included women with uncomplicated urogenital/rectal gonorrhoea ($n = 104$) who were subdivided into those with asymptomatic ($n = 56$) and those with symptomatic infections ($n = 48$); men with uncomplicated urogenital/rectal gonorrhoea ($n = 129$) who were subdivided into those with 1-4 days of symptoms ($n = 79$), those with 5-30 days of symptoms ($n = 34$), and those who fell into neither of these two groups ($n = 16$); patients ($n = 33$) with oropharyngeal gonorrhoea (27 of them had also urogenital/rectal infections); and patients with complicated gonococcal infections ($n = 11$). Six of the patients had a disseminated gonococcal infection, one Bartholinitis, three epididymitis, and one a rectal abscess.

Patients without gonorrhoea

This included women attending the clinic for a routine examination for STD ($n = 28$); prostitutes ($n = 21$); men attending the clinic for a routine examination for STD ($n = 72$); and men with non-gonococcal urethritis (NGU) ($n = 83$).

CONTROL SERA

Control sera were obtained from 119 blood donors (46 women, age range 19-65 years, and 73 men, age range 20-62 years) and 58 children (25 girls and 33 boys aged between 10 and 12 years). Sera of blood donors were kindly donated by Dr F Kothe of the Rotterdam Blood Transfusion Service, and sera from children by Professor Dr H A Valkenburg and Dr F Klein of the Department of Epidemiology, Erasmus University Rotterdam. The children were all healthy inhabitants of Zoetermeer (a suburb of The Hague).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Gonococcal pili of strain 1443 (Rotterdam) were purified according to the method of Robertson *et al.*⁸

The pilus antigen was sonicated for one minute at 0°C with an MSE ultrasonic disintegrator (8 μ , probe 3.3 mm) immediately before use. The microtitre plates (flat-bottom polyvinylchloride, Cooke Microtiter system) were coated by depositing 50 μ l pilus antigen (2-6 μ g protein per ml depending on the pilus batch) in each cup; incubation was carried out for four hours at 37°C in a water bath. (All other incubations were carried out under the same conditions.) The plates were washed once with PBS pH 7.8, 100 μ l 1% bovine serum albumin was added, and the plates re-incubated

for 30 minutes at 37°C in order to cover any free reaction sites. After being washed with PBS pH 7.8 the plates could be stored for up to 10 days at 4°C. Patient sera were diluted 1/100 in PBS pH 7.8, and 50 μ l aliquots of the diluted serum were added (in duplicate) to the cups and incubated for 45 minutes at 37°C. The plates were then washed four times with PBS pH 7.8 (containing 0.05% Tween 20), and 50 μ l of an optimum dilution of horseradish peroxidase-conjugated antihuman total immunoglobulin (Institut Pasteur) was added. The plates were then reincubated for 45 minutes at 37°C and washed six times with PBS-Tween pH 7.8. After addition of 50 μ l orthophenylenediamine-containing substrate the plates were incubated for 50 minutes at 37°C. The reaction was stopped by the addition of 50 μ l 2 mol/l H_2SO_4 . A positive and a negative serum were included on each microtitre plate. A row of blanks (coated cups without serum) and a maximum colour control (conjugate plus substrate) were likewise included on each plate.

All sera were tested only at a dilution of 1/100. Absorbance was read at 492 nm using a Multiskan (Titertek) spectrophotometer, with reference to the row of blanks. Readings were classified as follows: (A = absorbance) $A \geq 0.90$, positive; $A < 0.90$, negative; A between 0.80 and 0.90, doubtful; and A between 0.90 and 1.00, weakly positive. All sera from the children had absorbances below 0.90.

The coefficient of variation within the test (plate to plate) and from test to test (day to day) was less than 15%; only for very low absorbances was it more than 15%.

The first and second serum samples were tested in the same run: all sera, including the controls, were tested in duplicate.

INDIRECT HAEMAGGLUTINATION REACTION (IHA)

The IHA was carried out as described by Reimann and Lind⁹ using purified gonococcal pili of Buchanan's strain 2686 (USA) as antigen. Pilus antigen was purified according to the method of Hermodson *et al.*¹⁰ The titre of a given serum specimen was recorded as the reciprocal value of the lowest serum concentration giving a positive reaction. Titres ≥ 40 were considered to be positive.

GONOCOCCAL COMPLEMENT FIXATION TEST (GCFT)

The GCFT was performed as a mechanised microtechnique using Ames Autotiter instrument. Briefly, 0.05 ml inactivated serum was automatically added to the U-shaped cups of clear Conphar trays and diluted in two-fold dilutions from undiluted to 1/512 in 0.85% saline. Antigen (0.025 ml) and complement (0.05 ml) were then added to each cup and the reagents incubated overnight at 4°C. The next

morning 0.025 ml each of amboceptor and 2% v/v sheep red blood cells suspended in saline were automatically dispensed into each cup. The trays were shaken and incubated at 37°C for one hour. The titre was read as the highest dilution giving 100% inhibition of lysis, if the control cup showed 100% haemolysis. Sera showing less than 100% haemolysis in the control cup were regarded as anticomplementary. About 5% of the sera were anticomplementary and could not be read. The antigen used in the GCFT test was prepared from *Neisseria gonorrhoeae* strain 46695 (Oliver) by ultrasonic treatment of whole organisms suspended in saline.

STATISTICAL ANALYSIS

Fisher's test¹¹ was used to compare the percentages of positive results in two different patient groups; the two-tail probability was computed as proposed by Cox and Hinkley.¹² McNemar's test¹¹ was used to compare the percentages of positive results in two consecutive serum samples from the same patients. The coefficient κ ^{13 14} was used to measure the agreement between two serological tests; κ equals zero under statistical independence and κ equals one when there is perfect agreement.

DEFINITIONS

Sensitivity

$$= \frac{\text{diseased persons with positive tests}}{\text{all diseased persons tested}} \times 100\%$$

Specificity

$$= \frac{\text{non-diseased persons with negative tests}}{\text{all non-diseased persons tested}} \times 100\%$$

Results

UNCOMPLICATED GONORRHOEA

The percentages of positive sera in the ELISA, IHA, and GCFT in patients with uncomplicated gonorrhoea are given in table I. When the results of sera obtained 11-22 days later (D2) are compared with those obtained at the first attendance (D1), a nearly significant increase ($0.05 < P < 0.1$) in percentage of positive sera was observed only in the ELISA. No significant difference in sensitivity was found between the ELISA and the IHA. Both tests, however, were significantly more sensitive than the GCFT on D1 and D2 ($P < 0.01$). For women with asymptomatic infections ($n = 56$) comparison of sensitivities of the tests on sera collected on D2 with those on D1 showed no significant differences for any of the three tests in this group. The sensitivities of the ELISA and the IHA showed no significant differences. The difference between these tests and the GCFT, however, was again significant for sera obtained both on D1 and on D2 ($P < 0.01$).

For women with symptomatic infections the sensitivities of the ELISA and IHA differed significantly for sera obtained on D1 ($P < 0.05$) but not on D2; those of the ELISA and GCFT differed significantly for sera obtained on D2 ($P < 0.05$) but not on D1. Sensitivities of the IHA and GCFT differed significantly for sera obtained both on D1 and on D2 ($P < 0.02$). ELISA and IHA sensitivities were both higher in the group of asymptomatic than in that of symptomatic infected women. This was not the case with the GCFT; for sera obtained on D2 the sensitivity of the test in the asymptomatic women was lower than that in the symptomatic infected women (18% versus 27%). Only in the ELISA did the sensitivity of the two groups differ significantly for sera obtained on D1.

Of the sera obtained from men with uncomplicated gonorrhoea ($n = 129$) the sensitivity of the ELISA and

TABLE I Sensitivities of three serological tests for sera of patients with uncomplicated gonorrhoea

Patient groups	Sensitivity of following tests (%):					
	ELISA		IHA		GCFT	
	D1*	D2*	D1	D2	D1	D2
Women						
With current gonococcal infections (GC) ($n = 104$)	45	53	58	54	21	22
With asymptomatic uncomplicated urogenital/rectal GC ($n = 56$)	57	61	63	55	21	18
With symptomatic uncomplicated urogenital/rectal GC ($n = 48$)	31	44	52	52	21	27
Men						
With current GC ($n = 129$)	32	41	36	50	10	14
With uncomplicated urogenital/rectal GC with complaints for 1-4 days ($n = 79$)	25	34	27	39	4	10
With uncomplicated urogenital/rectal GC with complaints for 5-30 days ($n = 34$)	44	62	53	77	23	23

*D1 = date of first examination; D2 = 11-22 days after D1

ELISA = enzyme-linked immunosorbent assay; IHA = indirect haemagglutination assay; GCFT = gonococcal complement fixation test

the IHA performed on D2 sera was significantly higher than that of tests on D1 sera ($P < 0.05$). The difference in GCFT sensitivities was not significant. The sensitivities of the ELISA and the IHA did not differ significantly in the total group of men; both were significantly more sensitive than the GCFT ($P < 0.01$). For men with 1-4 days of symptoms ($n = 79$) the sensitivities of the tests on D1 and D2 sera were increased only in the IHA ($P < 0.01$). Comparison of the three tests with each other gives the same result as that in the total group of men.

Similarly, a comparison of the sensitivities of the tests on D2 sera with those on D1 sera showed a significant increase in the percentage of positive sera in both the ELISA ($P < 0.05$) and the IHA ($P < 0.01$). Only in this subgroup of men did the ELISA and IHA sensitivities differ significantly from each other on D2 sera ($P < 0.05$). The difference in sensitivity between the ELISA and the GCFT was significant only on D2 sera ($P < 0.01$). The IHA and GCFT differed significantly both on D1 and on D2 sera, as they did in the total group of men. A comparison of the sensitivity of the tests on sera from men with 1-4 days of symptoms with those from men with 5-30 days of symptoms showed that each test was more sensitive on sera from the latter subgroup of patients, the difference nearly always being significant.

OROPHARYNGEAL GONORRHOEA

Of the patients with gonococcal pharyngitis ($n = 33$), 27 also had urogenital/rectal infections. Serum from one patient was not tested by the GCFT. A second serum sample was obtained from 24 patients 6-22 days after D1. The ELISA, IHA, and GCFT sensitivities were 73%, 58%, and 53% respectively on D1 sera, the respective sensitivities on D2 sera being 96%, 75%, and 63%. The ELISA was the most sensitive test, being significantly more sensitive than the GCFT.

COMPLICATED GONOCOCCAL INFECTIONS

Of the 11 sera, nine (82%) were positive by the ELISA, nine by the IHA (90%, 10 sera were tested) and seven (63%) by the GCFT; 7-26 days later eight out of nine (89%) sera tested were positive by the ELISA, nine (100%) by the IHA, and six (67%) by the GCFT.

WITHOUT GONORRHOEA

The specificities of the three tests on sera from patients who did not have gonorrhoea are shown in table II. In women who attended for a routine examination for STD the ELISA and IHA specificities did not differ significantly. ELISA and GCFT showed a near significant difference in specificity only on D1 sera (75% versus 96%). IHA and GCFT results differed significantly both on D1 and on D2 sera (71% versus 96%) ($P < 0.05$). The specificities of all tests were lowest in sera from prostitutes ($n = 21$). Again, the ELISA and IHA did not differ significantly in specificity; both tests, however, were significantly less specific than the GCFT ($P < 0.05$).

In men reporting for a routine examination the specificities of the ELISA, IHA, and GCFT did not differ significantly. The specificity of the GCFT was higher than that of the IHA. Test specificity of sera from men with non-gonococcal urethritis (NGU) was slightly lower than that in the preceding group. In this group the difference between the ELISA and IHA on D1 sera just escaped significance (84% versus 75%). Only on D2 sera did the GCFT show a higher specificity than the ELISA ($P < 0.05$). The GCFT was significantly more specific than the IHA ($P < 0.01$). Comparison of the various groups showed that the only significant difference in ELISA specificity was found in sera from women attending for a routine examination and prostitutes on D2. The results obtained on D2 did not differ significantly from those on D1.

TABLE II Specificity of three serological tests for sera of patients without current gonococcal infections, blood donors, and children

	Specificity of following tests (%):					
	ELISA		IHA		GCFT	
	D1*	D2*	D1	D2	D1	D2
<i>Patients and controls</i>						
Women without current gonococcal infections (GC):						
Attending for routine examination ($n = 28$)	75	82	71	71	96	96
Prostitutes ($n = 21$)	52	38	52	48	86	91
Men without current GC:						
Attending for routine examination ($n = 72$)	87	90	82	82	93	96
With non-gonococcal urethritis ($n = 83$)	84	83	75	76	90	94
Blood donors ($n = 119$)	100		94		99	
Children aged 10-12 years ($n = 58$)	100		100		100	

*D1 = date of first examination; D2 = 11-22 days after D1

ELISA = enzyme-linked immunosorbent assay; IHA = indirect haemagglutination assay; GCFT = gonococcal complement-fixation test

The specificities of all three tests was high in the blood donors ($n = 119$) and children ($n = 58$).

DEGREE OF AGREEMENT BETWEEN THE TESTS

The agreement between the tests was measured using the coefficient of agreement κ and is shown in table III. When the two tests agree only by chance, the expected (n_c) and the observed (n_o) numbers were about equal. The ratio $(n_o - n_c)/(N - n_c)$ is known as the coefficient of agreement κ . For the agreement between the ELISA and GCFT, $\kappa = 0.44$ and for that between ELISA and IHA, $\kappa = 0.26$. Nearly the same result was obtained when the IHA and GCFT were compared, namely $\kappa = 0.20$.

Discussion

Many serological tests for the detection of antibodies to *Neisseria gonorrhoeae* have been developed. It is difficult, however, to compare the various results because the tests were performed under different conditions. The culture technique, the sites from which the cultures were taken, and the selection of patient populations differed widely in the different studies. Few studies have compared two or more serological tests in the same patient populations.^{6, 15-17} Danielsson *et al*¹⁷ compared a GCFT and an immunoelectrophoresis test (IE) in the same patient groups. The GCFT had sensitivities of 20-25% in men and 30-40% in women with uncomplicated gonorrhoea. The sensitivity of the IE was 24% in patients (male and female) with uncomplicated gonorrhoea. Reimann *et al*⁶ compared an IHA test using a mixture of pili from two Danish gonococcal strains as antigen with the GCFT (routine test in Denmark until 1979). The sensitivity of the IHA test was 70% for women and 59% for men. The GCFT sensitivity was about 9% in both groups.

We performed a comparative study of three tests: an ELISA and IHA, using two different gonococcal pili as antigen, and a GCFT using whole gonococcal cells as antigen. We found that sensitivities of the ELISA and IHA were highest in sera from patients with

complicated gonococcal infections and oropharyngeal gonorrhoea, in asymptomatically infected women, and in men with 5-30 days of symptoms of uncomplicated gonorrhoea. The sensitivity of the GCFT was very low in the uncomplicated infections. In uncomplicated gonorrhoea the highest sensitivity (27%) was obtained on sera collected on D2 (11-22 days after the first examination) for women with symptomatic infections. In oropharyngeal gonorrhoea the sensitivity of the ELISA was significantly higher than that of the GCFT, and nearly significantly higher than that of the IHA. Only in sera from symptomatic infected women and men with 5-30 days of symptoms of uncomplicated gonorrhoea did the ELISA and IHA differ significantly in sensitivity (IHA being superior). The ELISA generally was slightly more specific than the IHA, but its sensitivity was slightly lower except in oropharyngeal gonorrhoea, where it was superior to the IHA. Using the coefficient of agreement κ , we found a higher degree of agreement between the ELISA and IHA ($\kappa = 0.44$) than between each of these tests and the GCFT ($\kappa = 0.26$ and 0.20). The ELISA and IHA systems have almost the same sensitivity, but the coefficient of agreement in our study was nevertheless lower than might be expected. The unexpectedly low agreement may have been due to different antigenic determinants on the pilus antigens, but this can only partly explain the relatively low agreement. Another but improbable possibility is that different components of pili are available as antigen in each system. Reimann *et al*¹⁸ recently reported that antigenic heterogeneity of gonococcal pili exists if human sera are used in the test system. By testing sera from Dutch women with gonorrhoea it seemed that pili of strain 6650 (Rotterdam) had a broader antigenicity in the IHA than the pili of Buchanan's strain 2686. For women with uncomplicated gonorrhoea (same sera as in this study) Reimann *et al*¹⁸ obtained sensitivities of 66% and 71% on D1 and D2 respectively. Oranje (unpublished data), using an ELISA performed with pili of strain 6650 in the same patient population, obtained virtually the same results: 69% on D1 and 67% on D2.

TABLE III Statistical correlation* between three serological tests for the detection of gonococcal antibodies in the sera of 233 patients with uncomplicated gonorrhoea

Test	Result	IHA		Statistical correlation	GCFT		Statistical correlation
		+	-		+	-	
ELISA	+	146 (95)	50	$\kappa = 0.44$ SE = 0.04	59 (32)	137	$\kappa = 0.26$ SE = 0.04
	-	79	191 (140)		17	253 (226)	
IHA	+				60 (37)	165	$\kappa = 0.20$ SE = 0.03
	-				16	225 (202)	

+ = Positive; - = negative; SE = standard error

*Numbers given in brackets are those which can be expected under statistical independence

The two pilus tests are far more sensitive than the GCFT, but the specificity of the latter is higher in high-risk groups. The low specificity of the pilus tests in these groups was partly due to the large proportion of patients with a history of previous gonococcal infections. Buchanan *et al*,² Reimann *et al*,⁹ and ourselves have found that antibodies to gonococcal pili are mostly of the IgG classes and can therefore persist for a long time (sometimes for years). Not infrequently some patients attending STD clinics may have been treated inadvertently in the past. Culture will then be negative but the serological test may be positive. The persistence of antibodies to gonococcal pili and the unsupervised use of antibiotics also explain the very low specificity of the tests in prostitutes. Moreover, the sensitivity of the gonococcal culture technique is not 100%. For a proper evaluation of the sensitivity and the specificity of these serological tests the incidence of false-negative gonococcal culture results would be of great importance.

Our findings suggest that gonococcal pili constitute an excellent antigen, which may be improved by including antigenically different pilus antigens in the antigen preparation used or by replacing the antigen with a pilus which shares a greater number of antigenic determinants.

Because of antibodies persisting from an earlier infection, these pilus tests cannot differentiate between a previous and a current infection, and a positive serological reaction should therefore always be confirmed with positive cultures or positive clinical symptoms (complicated gonorrhoea) before the diagnosis of gonorrhoea is made. Because of their relatively high sensitivities the pilus tests might be suitable for case-detecting or screening.

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